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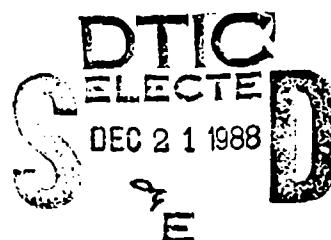
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REPORT DOCUMENTATION PAGE

AD-A202 503

DTIC FILE COPY

1b. RESTRICTIVE MARKINGS			
3. DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution is unlimited			
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE			
4. PERFORMING ORGANIZATION REPORT NUMBER(S) NMRI 85-118			
6a. NAME OF PERFORMING ORGANIZATION Naval Medical Research	6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION Naval Medical Command	
6c. ADDRESS (City, State, and ZIP Code) Bethesda, Maryland 20814-5055			
7b. ADDRESS (City, State, and ZIP Code) Department of the Navy Washington, D.C. 20372-5120			
8a. NAME OF FUNDING/SPONSORING ORGANIZATION Naval Medical Research and Development Command	8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER	
8c. ADDRESS (City, State, and ZIP Code) Bethesda, Maryland 20814-5055			
10. SOURCE OF FUNDING NUMBERS			
PROGRAM ELEMENT NO. N.A	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.
11. TITLE (Include Security Classification) Endotoxin interactions with platelets			
12. PERSONAL AUTHOR(S) Walker RI, Casey LC			
13a. TYPE OF REPORT Book chapter	13b. TIME COVERED FROM _____ TO _____	14. DATE OF REPORT (Year, Month, Day) 1985	15. PAGE COUNT 19
16. SUPPLEMENTARY NOTATION in: Handbook of Endotoxin. Volume 3: Cellular Biology of Endotoxin, Editor: L.J. Berry. Elsevier Science Publishers, 1985, pp. 225-243			
17. COSATI CODES		18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)	
FIELD	GROUP	SUB-GROUP	Immunology Platelet aggregation Endotoxemia Platelet membranes Serotonin Complement
19. ABSTRACT (Continue on reverse if necessary and identify by block number)			
20. DISTRIBUTION/AVAILABILITY OF ABSTRACT <input checked="" type="checkbox"/> UNCLASSIFIED/UNLIMITED <input type="checkbox"/> SAME AS RPT. <input type="checkbox"/> DTIC USERS		21. ABSTRACT SECURITY CLASSIFICATION Unclassified	
22a. NAME OF RESPONSIBLE INDIVIDUAL Phyllis Blum, Information Services Division		22b. TELEPHONE (Include Area Code) 202-295-2188	22c. OFFICE SYMBOL ISD/ADMIN/NMRI





Approved by	Mr. J. H. S. Smith	<input checked="" type="checkbox"/>
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CHAPTER 11

Endotoxin interactions with platelets

RICHARD I. WALKER AND LARRY C. CASEY

1. INTRODUCTION

Approximately 500 million years ago the horseshoe crab (*Limulus polyphemus*) evoked an antimicrobial defensive system consisting of a single cell type – the amebocyte – which degranulates to trap bacteria in a clot and subsequently destroy them. The antimicrobial system has become more specialized in mammals, but the teleologic descendant of the amebocyte – the platelet – is still important in the inflammatory response of the host to microorganisms. For example, platelets interact with pathogens and release mediators of inflammation, participate in trapping microorganisms for phagocytes and, although not bactericidal themselves, enhance the bactericidal effects of leukocytes (Clawson 1974; Clawson et al 1975; Smith 1972; Wilder and Lubin 1973).

The potentially beneficial effect of platelets on infection may become detrimental if thrombosis becomes too extensive to be resolved effectively. The host response to microorganisms can also be induced to self-destructive levels by challenge with purified endotoxin, a lipid-polysaccharide component of the cell walls of gram-negative bacteria. Within minutes after injection, most endotoxin in the bloodstream is associated with platelets (Braude 1964; Evans 1972). These cells have a profound influence on the rate of transport of endotoxin (Das et al 1973) and other particulate material (Donald 1972; Noyes et al 1959; Van Aken and Vreeken 1969) to the reticuloendothelial system. For example, plasma clearance of endotoxin is significantly slower in thrombocytopenic rabbits than in normal animals (Das et al 1973). In achieving this purpose, platelet-endotoxin complexes must pass through the capillary network of nonreticuloendothelial organs, a process which depends on maintenance of a balance between aggregation and disaggregation of platelets (Van Aken and Vreeken 1969).

Uncontrolled thrombosis in the microcirculation of major organs may be a key event during endotoxemia which could contribute to the diversity of host responses to endotoxin. This concept is consistent with microcinematographic observations by Urbaschek (1971). Within minutes after injection of endotoxin into rabbits, blood flow begins to slow and mast cells degranulate. Rouleaux formation of erythrocytes occurs, granulocytes adhere to the vessel walls and roll along the endothelium, and large platelet aggregates form and are partly disaggregated again. Soon irreversible platelet thrombi form, an event which could be significant to the further course of endotoxemia. Endothelial cells swell and together with

wall-adhering blood cells, intensify the narrowing of the vessel lumen. Blood flow continues to decrease, first in the venules and capillaries and later in the arterioles. By 1 hour after challenge stasis occurs, capillary wall permeability increases and microbleeding begins.

2. GENERAL CHARACTERISTICS OF THE PLATELET RESPONSE TO ENDOTOXIN

The platelet response to endotoxin was recognized in the 1950s. Following *in vivo* administration of endotoxin there is platelet aggregation (Stetson, 1951) and increased plasma serotonin and histamine (Shimamoto 1958). Temperature affected both the aggregation and serotonin release (Des Prez et al 1961). At 4°C no change was observed in either aggregation or serotonin release, whereas at 37°C there was time-dependent serotonin release and aggregation. Furthermore, the clotting time of platelet-rich plasma was shortened following the addition of endotoxin (Des Prez 1964), and this could be related to the fact that platelets contribute a phospholipid called platelet factor 3 to the coagulation system (Horowitz and Spaet 1961). The clotting times of both platelet-rich plasma and whole blood were significantly shortened by the addition of endotoxin, but the phenomenon can be reversed by removing the platelets (Horowitz et al 1962; McKay et al 1958). Horowitz demonstrated that by lysing the platelets with detergent and then adding phospholipid (brain cephalin), the coagulation time could be shortened. Adding endotoxin did not further shorten the coagulation time once the platelets had been lysed or cephalin had been added.

Preincubation of antigen with immune plasma prior to addition of platelets and anticoagulant initiated effects similar to the endotoxin-platelet interaction (Des Prez 1964). The antigen-antibody reaction probably activates complement, and this complex could then react with platelets as a particle might. Movat et al (1965) and Spielvogel (1967) suggest that the antigen-antibody-platelet and platelet-endotoxin complexes behave as particles and that platelet injury is associated with phagocytosis. Movat showed that platelets ingest latex particles, ferritin-anti-ferritin complexes, and Spielvogel showed the ingestion of endotoxin by platelets. These particle ingestions by the platelets released serotonin into the plasma.

The similarity in platelet-induced injury by endotoxin and that induced by antigen-antibody reactions led Des Prez and Bryant (1966) to study the divalent cation requirements for these two types of reactions. To document that platelet injuries were similar to immune reactions, they compared endotoxin-induced release of serotonin with serotonin release induced by antigen added to platelets derived from immunized animals. With various concentrations of anticoagulants in platelet-rich plasma (heparin, 100 µg/ml; sodium citrate, 0.38%; sodium EDTA, 0.1% or 0.17%) Des Prez and Bryant (1966) showed that endotoxin caused release of serotonin when 0.38% sodium citrate or 0.1% sodium EDTA was present, while antigen-antibody did not. When the concentration of EDTA was increased to

0.17%, neither endotoxin nor antigen-antibody caused serotonin release. This difference was not observed when antigen and platelet-poor plasma were allowed to interact in the absence of chelating agents. When platelets and citrate were added following the incubation, the release of serotonin was similar to that induced by endotoxin. Des Prez and Bryant (1966) showed that the reason for the initial differences between endotoxin and antigen-antibody complex interactions with platelets were related to the divalent cation concentrations (Ca^{2+} and Mg^{2+}), and that more divalent cations were necessary for the antigen-antibody reactions than for endotoxin reactions in the presence of the same concentration of chelating agents. More recently, the noncytotoxic secretory activity of platelets has been shown to have a specific requirement for calcium (Morrison et al 1980).

Differences were also noted between platelet injury caused by thrombin and that induced by endotoxin (Des Prez 1964). The lag period of serotonin release and of aggregation led Des Prez to think that the effect of endotoxin on platelets was indirect, possibly secondary to the action of a proteolytic enzyme, because platelet-rich plasma incubated with trypsin and no calcium did not decrease the fibrinogen level, whereas with trypsin and calcium, lower fibrinogen values and increased serotonin levels in the plasma were seen, even though no aggregation occurred. Using thrombin-induced platelet injury, Des Prez compared it to endotoxin-induced platelet injury. Very small amounts of thrombin induced platelet injury, and this reaction was immediate and non-progressive as was induced clot formation. In contrast to endotoxin, concentrations of thrombin sufficient to produce gross clot formation in citrated plasma were less than those required to induce serotonin release from the platelets. Small doses of heparin prevented thrombin injury to platelets, whereas very large amounts of heparin were required to prevent endotoxin-induced platelet injury.

3. COMPONENTS OF THE PLATELET-ENDOTOXIN INTERACTION

Since the early studies of platelet aggregation and release responses, a variety of humoral and cellular elements have been identified as important to the platelet-endotoxin interaction.

3.1. Plasma factors

Des Prez et al (1961) showed that heating plasma to 56°C for 30 minutes before adding the platelets and endotoxin, inactivated the serotonin release and the aggregation. This suggested that there is a heat-labile plasma factor necessary to mediate the effects of endotoxin on platelets. This factor, which had earlier been detected by McKay et al (1958), was of considerable interest because of its possible role in the immune mechanism and the coagulation system. With an intricate set of experiments, Ream et al (1965) showed not only that plasma and platelets were required for endotoxin to induce platelet injury, but also that the plasma factor could be better characterized. Heating the endotoxin to 56°C did not alter its effect on

platelets, but heating the plasma for 2 minutes at 56°C inactivated the factor necessary for the platelet-endotoxin interaction.

Ream et al (1965) found evidence that a plasma factor necessary for the aggregation of platelets might be Factor V. By removing platelet-rich plasma from humans with known individual factor deficiencies, aggregation was observed after the addition of endotoxin in every case except with the plasma deficient in Factor V. If Factor V was added, the aggregation occurred. However, if the Factor V was allowed to be below 30% of normal (by dilution technique), aggregation did not occur following exposure to endotoxin (100 µg/ml). To simulate the lipopolysaccharide of endotoxin, several different fatty acids were added individually to platelet-rich plasma (normal), but no aggregation was observed. With rabbit platelet-rich plasma, endotoxin caused aggregation predictably, but with human platelet-rich plasma and endotoxin, the aggregation was variable. Additionally, Ream showed that contact Factor XII, believed to initiate the clotting mechanism, was not involved in the platelet-endotoxin interaction since platelet-rich plasma deficient in Factor XII allowed aggregation to occur when exposed to endotoxin. Müller-Berghaus and Schneberger (1971) confirmed that Factor XII (Hageman factor) was not involved in the platelet-endotoxin interaction because a single dose of endotoxin (10-20 µg/ml) did not decrease the amount of this factor. Since pretreatment with one of the coumarin drugs prevented the fall of the level of Hageman factor after the injection of thorotrust or endotoxin, Müller-Berghaus concluded that the contact factor was not the trigger mechanism in coagulation from the endotoxin-platelet interaction.

3.2. Complement

Even though Ream et al (1965) had demonstrated that the heat-labile plasma factor necessary for the platelet-endotoxin reaction could be Factor V, Des Prez (1967) was not satisfied. He demonstrated (a) that whole-blood complement titers were not decreased during the course of endotoxin-platelet injury in recalcified citrated plasma and (b) that plasma adsorbed with zymosan at 16°C in the presence of EDTA did not lower the complement titers and it was furthermore similar to heated plasma (56°C for 30 minutes) in failing to support the platelet-endotoxin interaction, and (c) the factor or factors removed or inactivated by zymosan and heating the plasma appeared to be similar in the amounts necessary to support the platelet-endotoxin interaction. Since Boyden (1966) had demonstrated that macroglobulin antibodies, reactive with the polysaccharide determinants of endotoxin of gram-negative bacteria, exist in all species virtually from birth, Des Prez speculated that the heat-labile plasma factor was a naturally occurring antibody.

Support for the fact that complement is involved in the platelet-endotoxin interaction was obtained by Gilbert and Braude (1962) who showed a decrease in serum complement after endotoxin injection into rabbits. Spielvogel (1967) demonstrated that the effects of endotoxin on rabbit platelets *in vitro* were due to the immune adherence phenomenon and the heat-labile plasma factor involved was complement. He found that measures known to inactivate some complement

components (heating, NH₄OH, zymosan) also prevented degranulation of rabbit platelets in citrated platelet-rich plasma. It was also shown that loss of granules in the platelets was associated with adherence of endotoxin particles to the platelet membrane. In primate blood (in which immune adherence receptor sites are on the red blood cells rather than on the platelets), platelet damage was not observed.

Gewurz et al (1968) investigated the interaction of lipopolysaccharides with C3-C9 of the complement system and found that each of the six components was consumed during these incubations, even with small amounts (10-25 µg/ml) of endotoxin. Interestingly, he showed that lipopolysaccharide consumed greater quantities of C3-C9 than did anti-human gamma globulin. Lipopolysaccharide reacting with hyperimmune rabbit serum leads to a complement consumption profile similar to that induced by the immune complexes. When highly purified preparations of any of the six terminal complement components were incubated with lipopolysaccharide, no consumption occurred. This indicated other serum factors were needed. Gewurz concluded that the complement system was involved in the biological actions of lipopolysaccharide.

Kane et al (1973) studied the interactions of the classical and alternative complement pathways with endotoxin lipopolysaccharide with special emphasis on platelets and coagulation. Using C4-deficient guinea pigs, known to have a complete block in the activity of the classical complement pathway, but with the alternative pathway intact, he demonstrated that there was no fall in the platelet count as seen in the normal animals following endotoxin injection. The alternative pathway was activated in C4-deficient guinea pigs since the C3-C9 titers did decrease. When C4 was restored in the C4-deficient animals, thrombocytopenia did occur as in the controls. Kane concluded that the classical and alternative complement pathways were required for thrombocytopenia and shortening of the clotting time.

Brown and Lachmann (1973) studied the interactions of complement and platelets in lethal endotoxin shock in rabbits. With radioactive chromium-labeled platelets in normal, in C6-deficient, and C3-C9-depleted rabbits, they were able to study the effects of the potentially lethal dose of endotoxin on survival and platelet sequestration. In the normal rabbits, the platelets were acutely destroyed with only a small portion returning to the circulation and all animals died. In the C3-C9-depleted animals, platelets survived normally following endotoxin injection and none of the animals died. In C6-deficient rabbits, platelets were acutely lost from the circulation, but were destroyed only variably. C6-deficient rabbits in which the platelet recovery was greater than 65% lived, whereas when the platelet recovery was less than 45%, the animals died. Platelet factor 3 activation by endotoxin was also studied in these animals both *in vitro* and *in vivo*. Marked activation of platelet factor 3 occurred in normal rabbits, minor activation in C6-depleted rabbits, and no activation in C3-C9-depleted rabbits. Brown and Lachman concluded that complement depletion markedly alters the platelet response to endotoxin. Depletion of C3-C9 appears to be absolutely protective and correlates well with absence of mortality, normal platelet survival, and failure to activate platelet factor 3.

Morrison and Oades (1979) showed that different portions of the endotoxin molecule activate different complement pathways. Activation of the alternative pathway is essential for platelet lysis, and preparations containing lipid A activate the classical pathway which does not cause lysis. Up to 5 µg of lipid A per ml of rabbit platelet-rich plasma did not induce aggregation in vitro, although aggregation of the same platelet preparation could be accomplished with a comparable amount of whole endotoxin (Walker and Beasley 1980). Lipid A attaches the endotoxin to the platelet membrane, and this attachment is enhanced by lipid A protein (Morrison and Oades 1979). The alternative pathway requires the polysaccharide region of the molecule, but lysis is not obtained if the lipid A portion is not available for platelet-endotoxin binding. Perhaps lipid A attachment to platelet membranes is necessary to bring the carbohydrate portion of the molecule into proximity with the membrane surface, where the alternative complement pathway is initiated.

Evidence that both lipid A and polysaccharide components of endotoxin are necessary for lysis or aggregation of platelets is shown by endotoxin detoxification experiments. Polymyxin B binds to lipid A, the toxic moiety of the endotoxin complex, thereby detoxifying the endotoxin preparation (Morrison and Jacobs 1976). This detoxified preparation also loses its platelet lytic capability. Similarly, megarad doses of gamma radiation ^{60}Co destroy fatty acid groups on lipid A (L. Bertok, personal communication) and thereby reduce the toxicity of the molecule (Previte et al 1967). Irradiated endotoxin no longer induces platelet aggregation (Walker et al 1983).

3.3. Specific antibodies

Lethal amounts of endotoxin may disturb the disaggregation process in non-reticuloendothelial organs, thereby causing abnormal deposition of platelet-endotoxin complexes which could have locally damaging effects. Persistence of endotoxin-platelet aggregates in the vasculature of nonreticuloendothelial organs previously has been associated with toxicity (Carey et al 1958; Noyes et al 1959), and animals made tolerant to the lethal effects of endotoxin demonstrate improved reticuloendothelial clearance of the toxin (Carey et al 1958; Herring et al 1963). Platelet responses to endotoxin may be an important factor in such improved clearance by the reticuloendothelial system.

We tested the hypothesis that platelets from tolerant rabbits have different aggregation characteristics than platelets of animals that are not resistant to the lethal effects of endotoxin (Walker et al 1978). Adult New Zealand white rabbits (2.5 kg) were made tolerant to *Salmonella typhi* endotoxin (lipopolysaccharide W, Difeo) by receiving 5 daily intraperitoneal injections of the toxin (0.2, 0.3, 0.4, 0.5 and 0.5 mg). The effectiveness of this regimen was determined by intravenous challenge of the rabbits with a lethal dose of endotoxin (0.15 mg/kg). Animals that had been made tolerant survived this dose of toxin.

Aggregation of platelets was determined on 1 ml samples of platelet-rich plasma

in plastic cuvettes equipped with plastic-covered magnetic stirring disks. Increased light transmission (aggregation) was recorded from a Beckman DU-2 spectrophotometer equipped with a magnetic stirrer and 37°C water bath. The pattern of the aggregation was followed. When the pen never returned toward the base line, aggregation was termed 'secondary' or 'irreversible'. Tracings in which the recorder pen returned toward the base line after reaching a maximum were termed 'primary' or 'reversible' aggregations.

Platelets from rabbits made tolerant to the lethal effects of endotoxin have aggregation characteristics different from those of platelets from nontolerant animals. Platelets from tolerant rabbits responded more rapidly to the aggregating effects of endotoxin than did platelets from nontolerant animals. Furthermore, unlike platelets from nontolerant rabbits, platelets from tolerant rabbits aggregated reversibly. These characteristics of platelets from tolerant rabbits may enhance reticuloendothelial clearance of endotoxin and thereby promote survival.

Since platelet-free plasma from tolerant rabbits could confer tolerant characteristics on platelets from nontolerant animals, a humoral factor must be principally responsible for altered platelet responsiveness to endotoxin. The specificity of the humoral factor was suggested by the observation that induction of 'tolerant' responses to endotoxin did not affect the aggregation responses of rabbit platelets exposed to ADP or collagen (Walker et al 1980). The time at which this humoral factor appeared in the plasma (1 week after the first injection used to induce tolerance) correlated with the appearance of antipolysaccharide (but not anti-lipid A) antibodies in the plasma (Walker and Beasley 1980). Furthermore, when specific anti-O antisera were added to normal platelet rich plasma, tolerant-like responses were observed following addition of endotoxin. Addition of anti-lipid A did not accelerate aggregation or disaggregation after exposure of platelets to complete endotoxin.

Greisman reported that antisera induced by smooth endotoxin are directed against the O-specific side chains (Greisman et al 1973). This is consistent with our finding (Walker and Beasley 1980) that plasma from tolerant rabbits or specific antisera reacted only with the smooth endotoxin and not with purified lipid A. Likewise, antisera against lipid A did not interact immunologically with smooth endotoxin.

Bult and Herman (1979) obtained responses with guinea pig platelets which were similar to those described above for rabbits made tolerant to the toxin. They found that pretreatment of guinea pigs with endotoxin caused a more rapid onset of the aggregation response when endotoxin was subsequently added to platelet-rich plasma from these animals. This effect was due to the presence of a humoral factor directed against the polysaccharide component of the endotoxin.

Lipid A did not initiate platelet aggregation unless its specific antibody was also present (Walker and Beasley 1980). This observation, which is consistent with a report that platelet lysis is not initiated by lipid A alone (Morrison and Oades 1979), provides further insight into the platelet-endotoxin interaction. Platelets have specific membrane receptor sites (Ausprung and Das 1978; Hawiger et al

1975; Washida 1978) to which lipid A can attach. However, complement is also involved in the rabbit platelet-endotoxin interaction (Spielvogel 1967), and depletion of these components correlates well with reduction of mortality of the animal and enhanced platelet survival. It now appears that lipid A serves as an attachment mechanism which brings the polysaccharide portion of the molecule into proximity with the platelet membrane where alternative complement components are assembled, thereby inducing visible platelet reactivity (Morrison and Oades 1979). In the tolerant animal the polysaccharide portion of the endotoxin is apparently modified by antibody, and this process could account for the altered platelet responsiveness to endotoxin seen in tolerant rabbits.

3.4. Prostaglandins

Prostaglandins are important mediators of platelet function and may contribute to the fate of these cells during endotoxemia. Recent work has demonstrated that the two endoperoxides derived from arachidonic acid, PGG₂ and PGH₂, are very potent in inducing rapid and irreversible aggregation of human platelets (Hamberg and Samuelsson 1974; Hamberg et al 1975). Acetylsalicylic acid, an inhibitor of cyclooxygenase and thus an inhibitor of endoperoxide formation, blocks secondary platelet aggregation, which suggests that the endoperoxides play a role in the platelet release reaction (Hamberg et al 1975). The addition of PGH₂ to platelet-rich plasma leads to rapid changes in shape, aggregation, and secretion of 5-hydroxytryptamine from the platelets. This phenomenon appears to be partly due to isomerization of the endoperoxides to thromboxane A₂ (TXA₂) by the enzyme thromboxane synthetase (Hamberg et al 1975).

The relative potencies of the prostaglandin endoperoxides and TXA₂ as platelet-aggregating agents are still unknown, but there is some evidence to suggest that TXA₂ is more potent (Needleman et al 1976). In contrast to the effects of endoperoxide on platelets, TXA₂ is at least 100 times more potent than PGH₂, as measured by the induction of contraction of aortic smooth muscle (Needleman et al 1976). The generation of TXA₂ during platelet aggregation *in vivo* may lead to amplification of the aggregation response as well as to local vasoconstriction. Butler et al (1982) demonstrated that a selective thromboxane synthetase inhibitor (*N*[7-carboxyheptyl]imidazole) failed to inhibit endotoxin-induced platelet aggregation, but did prolong the bleeding time. The prolongations of the bleeding time may be the result of the isomerization of the unconverted endoperoxides into prostacyclin (PGI₂) (Nijkamp et al 1977; Vermylen et al 1981), possibly by endothelial cells (Gryglewski et al 1976). These results may suggest that the most important role of TXA₂ in hemostasis is not as an amplifier of platelet aggregation, but rather as a potent vasoconstrictor which, by making the vessel smaller, facilitates hemostasis at the site of vessel injury.

Thromboxane may contribute to the pathophysiology of endotoxin shock. Cook et al (1981a) provided strong evidence for the importance of thromboxane in rat endotoxin shock by demonstrating that essential fatty acid-deficiency in rats, as

well as the pretreatment of rats with either a cyclooxygenase inhibitor or a thromboxane receptor antagonist, all improved survival in a lethal model of endotoxin shock.

Furthermore, prostacyclin, which has the opposite effects of thromboxane (inhibits platelet aggregation and is a vasodilator), is beneficial in treating endotoxin shock (Fletcher and Ramwell 1980; Lefer et al 1980). For example, in dogs challenged with *Escherichia coli* endotoxin (1 mg/kg) and treated with a continuous infusion of prostacyclin (20 ng/kg/min) from 15 minutes before to 4 hours after the administration of the endotoxin, survival was increased from 42% to 83%. This effect must be due to factors other than the inhibition of platelet aggregation since prostacyclin has no effect on direct endotoxin-induced platelet aggregation and does not prevent the *in vivo* endotoxin-induced thrombocytopenia (Fletcher and Ramwell 1980).

The importance of thromboxane in primate endotoxin shock appears to be different than in rat endotoxin shock, as the pretreatment of baboons with either OKY 1581 (a selective thromboxane synthetase inhibitor) or imidazole fails to improve survival (Casey et al 1982). In addition, these drugs do not prevent the endotoxin-induced thrombocytopenia. However, it is clear that endotoxin-induced pulmonary hypertension is mediated by increased TXA₂ formation (Casey et al 1982), and not by the mechanical occlusion of pulmonary vessels by either platelet or white blood cell aggregates as previously suggested (Pennington et al 1973).

Since a large number of tissues and cells are capable of producing thromboxane, the source of the increased plasma thromboxane after intravenous endotoxin administration remains unclear. When endotoxin is injected into rabbits, their platelets develop an increased capacity to synthesize thromboxane (Prancan et al 1981). All thromboxane may not come from the platelets, however, because in sheep or baboons, the increase in plasma thromboxane occurs immediately after the injection of the endotoxin, whereas the decrease in platelet count occurs over several hours (Harris et al 1980; Smith et al 1981). The incubation of neutrophils *in vitro* with endotoxin will stimulate them to produce thromboxane which can then cause platelet aggregation (Spagnuolo et al 1980). In addition, neutropenia decreases endotoxin-induced increases in plasma thromboxane (Hüttemeier et al 1982).

3.5. Other cell types affecting platelets

Direct and indirect effects of endotoxin on platelets may be altered by responses of other cell types which interact with the toxin. These cells include macrophages, polymorphonuclear leukocytes and endothelial cells.

Macrophages are important in the detoxification of endotoxin (Filkins 1971) and regulation of many host responses to the toxin (Berry 1972). The importance of these cells to survival during endotoxemia is indicated by the increased resistance to the lethal effects of endotoxin obtained in normal strains of mice receiving bone marrow transplants from genetically resistant animals (Michalek et al 1980). Ma-

crophages from genetically resistant mice (Sultzter and Goodman 1977) or tolerant mice (Snyder et al 1979) release lower amounts of mediators during phagocytosis, and this reduced responsiveness to endotoxin stimuli may diminish the degree of inflammatory responses obtained. For example, cultured mouse peritoneal macrophages secrete factors which cause rabbit platelets to aggregate and release serotonin (Blumenthal et al 1980). The nature of these factors is unknown, but macrophage preparations are known to secrete a variety of prostaglandins in response to inflammatory stimuli (Humes et al 1977) which include prostacyclin and TXA₂ (Krausz et al 1981; Cook et al 1981b). Reduction of such release could reduce microcirculatory damage. Additionally, resistant macrophages may secrete endotoxin-detoxifying substances or regulators of thrombosis, which would also reduce damage in the microcirculation.

Polymorphonuclear leukocytes could help reduce platelet aggregation. Tolerant animals mobilize granulocytes more effectively than normal animals (Fruhman 1972), and these cells may work with platelets in the delivery of the toxin to the reticuloendothelial system. Granulocytes are attracted to platelet thrombi and participate in their subsequent resolution through phagocytosis (Shirasawa and Chandler 1971) and release of mediators (Canoso et al 1974; Levine et al 1976; Rodvien et al 1976; Wautier et al 1976). Hydrogen peroxide is a leukocytic mediator released during phagocytosis (Iyer et al 1961; Root et al 1975) which reduces aggregation induced by ADP and other nonmicrobial substances (Canoso et al 1974; Levine et al 1976; Rodvien et al 1976). However, this substance has no effect on platelet aggregation induced by direct interaction with endotoxin (Walker et al 1980). This type of system seems well suited for elimination of microorganisms with simultaneous reduction of the dangerous additional aggregation induced by exposed collagen, ADP and other host factors.

Endotoxin can directly injure endothelial cell membranes with resulting intracellular edema and formation of gaps between cells which permit extrusion of erythrocytes (Batis et al 1980). In vivo endotoxin-dependent granulocyte and complement-mediated events could also damage endothelial cells (Sacks et al 1978). Subendothelium exposed by endothelial cell detachment permits platelet adhesion and various degrees of activation which may be important in the pathogenesis of the thrombotic complications of endotoxemia.

4. ENDOTOXIN INTERACTION WITH PRIMATE PLATELETS

Direct effects of endotoxin on primate platelets have been difficult to demonstrate in vitro. For example, aggregation responses readily observed in rabbit or dog platelet-rich plasma exposed to endotoxin are not obtained in primate preparations. However, thrombocytopenia does occur in primate models challenged with endotoxin (J.R. Fletcher, unpublished data). Male baboons weighing 20-30 kg were restrained and subjected to an LD₅₀ dose of *E. coli* endotoxin (3 mg/kg given as an intravenous bolus via a peripheral vein). By 15 minutes after challenge,

circulating platelets in those animals dropped to approximately 50% of normal values. Furthermore, when the responsiveness of the remaining platelets to ADP (2 µg/ml) was tested, they were found to be refractory to the induction of aggregation.

In 1965 Ream et al reported aggregation of washed human platelets in the presence of endotoxin and a heat-labile plasma factor. In general, however, relatively little success has been obtained in studies of platelet-endotoxin interactions in humans and other primates.

Das et al (1973) used the Limulus test to show that human platelets do interact with bacterial endotoxin. The sensitive component of the human platelet membrane interacts with endotoxin with subsequent release of adenine nucleotides and platelet factor 3 (Hawiger et al 1977). This receptor is apparently specific for endotoxin because zymosan does not release platelet factors as endotoxin does (Hawiger et al 1975). These authors go on to suggest that there is not only a specific endotoxin-sensitive component on human platelets, but also an indirect pathway of activation in which microbial antigens complexed with antibody and/or complement interact with platelets through receptors for the IgG Fc fragment and complement.

As Ream's work indicated (1965), plasma proteins are very important in the primate platelet-endotoxin interaction. In a plasma-free medium endotoxin, complexed with copper, adhered to human platelet surfaces and caused breaks in the membrane, pseudopod formation and centralization of platelet organelles (Ausprung and Das 1978). Normal plasma interferes with this reaction. An interesting observation is that the copper-containing protein ceruloplasmin, which becomes elevated prior to thrombocytopenia associated with gram-negative infection, may potentiate adsorption of blood-borne endotoxin by human platelets.

Platelet interaction with endotoxin may initiate a signal to the host that bacteria are present, thereby inducing an inflammatory response. With this in mind, we tested (Sheil and Walker 1979) the hypothesis that human platelets may influence endotoxin-induced inflammation through alteration of the chemotactic responsiveness of polymorphonuclear leukocytes. Leukocyte-rich preparations were placed on filters in chemotaxis chambers and exposed to platelet-rich or platelet-free human plasma before and after activation of the plasma with *Salmonella typhi* (Westphal preparation - Difeo) endotoxin.

With the addition of 25 ng - 0.5 µg of endotoxin to a milliliter of human platelet-rich plasma we observed an increase in leukocyte chemotaxis of 50-85% above that seen in unactivated platelet-rich plasma. However, activation of platelet-rich plasma with 1 and 2 µg of endotoxin resulted in an increased polymorphonuclear leukocyte chemotactic response of only 30-45%. These responses were significantly lower ($P < 0.01$) than the responses obtained with 1 and 2 µg of endotoxin in platelet-poor plasma. With the addition of 5, 10 and 100 µg of endotoxin, chemotaxis in response to activated platelet-rich plasma was increased by 75-100%.

Although caution must be exercised in interpreting these in vitro findings, obser-

vations made *in vivo* by others indicate that impairment of chemotactic responsiveness is a phenomenon associated with endotoxemia (Berthrong and Cluff 1953; Cluff 1953; Territo and Golde 1976). Furthermore, impairment of chemotaxis is associated with larger doses of endotoxin rather than with smaller doses of the toxin (Fruhman 1972). We can also make an association between platelet effects on leukocyte chemotaxis and survival from endotoxemia. Treatment of endotoxin with CrCl₃ reduces some toxic properties of the endotoxin molecule (Prigal et al 1973; Snyder et al 1978). Therefore, it is of interest that the reduction in chemotaxis seen in response to platelet-rich plasma activated with 1 and 2 µg of untreated endotoxin does not occur with Cr-treated endotoxin (Walker et al 1980).

5. POSSIBLE SIGNIFICANCE OF PLATELET INJURY DURING ENDOTOXEMIA

Microcirculatory failure may be a critical event during endotoxemia. The importance of events at this site is suggested by studies of the resolution of endotoxin-induced damage in the microcirculation of C3HeB/FeJ mice and the closely related, but endotoxin-resistant, C3H/HeJ mice (Walker and Parker 1980). These animals were challenged intraperitoneally with 1 mg of *Salmonella typhi* endotoxin, and early neutrophil sequestration in the pulmonary vasculature observed microscopically. In the C3H/HeJ group this neutrophil sequestration was apparent at 1 hour after challenge; it began to decline in incidence by 3 hours and was back to nearly normal levels by 6 hours. Although initial neutrophil sequestration in C3HeB/FeJ mice was similar to that of the C3H/HeJ mice, by 3 hours neutrophil sequestration became extensive and persisted at 6 hours in the C3HeB/FeJ mice in contrast to the near-absence of sequestration in the lungs of C3H/HeJ mice at this time. This difference in resolution of inflammation in the microcirculation may be associated with the finding that the challenge dose killed most of the sensitive but none of the resistant mice.

In later studies (Walker et al 1982), lung parenchymal tissue was excised from resistant and sensitive (this time C3H/HeN) mice 6 hours after challenge with 0.8 mg of endotoxin, and the *in vitro* production of TXA₂ or PGI₂ over a 30-minute period was determined by radioimmunoassay. TXA₂ and PGI₂ released by lung fragments taken 6 hours after challenge with endotoxin did not differ from normal levels of these substances in C3H/HeN mice. In contrast, TXA₂ release was normal at 6 hours in C3H/HeJ mice, but PGI₂ was significantly increased. The ratio of PGI₂/TXA₂ in resistant mice was about twice that seen in sensitive mice. Since PGI₂ can reduce leukocyte adhesion, an increase in the ratio of this substance to TXA₂ may be associated with resolution of polymorphonuclear leukocytes from the pulmonary microcirculation of endotoxin-resistant mice.

These microcirculatory disturbances can both affect platelets and be affected by them. Direct injury to platelets may be important with large doses of endotoxin. Cellular targets of endotoxin such as platelets can be expected to respond to the

toxin in a dose-dependent manner. This type of response has been obtained in vitro with dog platelets which aggregate in greater numbers as the concentration of *Serratia marcescens* endotoxin is increased from 25 ng to 5 µg/ml (Fletcher and Ramwell 1980). In vivo dose effects of endotoxin on dog platelets have also been observed (Walker 1980). Small (4 µg/kg) doses of *S. typhi* endotoxin administered intravenously produce no thrombocytopenia, but large, sublethal (1.8 mg/kg) doses of endotoxin induced marked thrombocytopenia.

That effects due to platelet injury can be critical during endotoxemia is indicated by studies in several models. Ream (1965) found a positive correlation between toxicity of different endotoxin preparations (as measured by chick embryo lethality) and their ability to induce aggregation of washed human platelets. Further correlation of the degree of endotoxin interaction with platelets and lethality was obtained by Washida (1978). He found that the LD₅₀ dose of endotoxin is 100 times less for newborn rabbits than for adult animals (50 µg/kg vs 5000 µg/kg). This increased sensitivity was associated with increased numbers of receptor sites for endotoxin on platelet membranes of newborn rabbits.

A significant contribution of platelets to host responses to endotoxemia is also indicated by studies which show that reduction of platelet aggregation increases survival from endotoxemia. A splenic extract which inhibits mouse platelet aggregation in vivo and in vitro also protects these animals from endotoxin-induced lethality (Spillert et al 1980). Lewis and Mustard (1969) found that inhibition of platelet aggregation during endotoxin shock prevents acute effects in adult rabbits. Furthermore, they obtained platelet aggregation and death similar to that seen during endotoxin shock infusion of ADP.

Studies in other species of animals indicate that platelet-endotoxin interactions may not be determinative during endotoxin shock. For example, in dogs made thrombocytopenic by estrogen pretreatment, mortality and gross necropsy findings following challenge with endotoxin were similar to animals with normal platelet levels that were also challenged with endotoxin administered intravenously (From et al 1976). Levin and Cluff (1965) showed that platelets do not contribute significantly to tissue damage induced by endotoxins; they selectively depleted rabbits of circulating platelets by treatment with guinea pig anti-rabbit platelet antibody. This thrombocytopenia did not prevent the cutaneous hemorrhagic lesion of the localized Shwartzman reaction following administration of endotoxin.

Further evidence that direct endotoxin-platelet interactions are not determinative in survival from endotoxin shock has been reported by Morrison et al (1980). They found that platelets from both endotoxin-resistant C3H/HeJ mice and responsive C3H/St mice manifest a similar time-dependent increase in calcium-dependent secretion following incubation with endotoxin.

Petschow (1983) showed that over 85% of endotoxin administered subcutaneously to mice remained at the site of injection, but lethality was still obtained. Unfortunately, no study of thrombocytopenia or other microcirculatory events accompanied these data. However, the data indicate that mediated effects are important in endotoxemia, and if thrombocytopenia is involved, it must be due to

indirect effects of host-generated factors. This may be one reason why factors such as PGI₂ which do not alter endotoxin-induced platelet aggregation are effective in enhancing survival following endotoxin challenge.

6. CONCLUSIONS

Endotoxin interacts rapidly with platelet membranes and, within minutes after experimental inoculation into animals, most is associated with the platelet fraction of the buffy coat. The endotoxin-platelet interaction contributes to inflammation by formation of aggregates and release of factors such as platelet factor 3, nucleotides, and serotonin. Also platelets may facilitate delivery of the toxin to the liver and spleen.

Most visible endotoxin effects are seen on platelets from subprimate animals which have immune adherence receptor sites. The initial part of this reaction involves the lipid region of the molecule and is followed by assembly of alternative complement pathway components on the polysaccharide portion of the molecule. This latter interaction causes platelet lysis. The presence of antibody to the O-polysaccharide region of the toxin alters the rabbit platelet response, so that aggregation occurs more rapidly and reversibly.

Primate platelets lack immune adherence receptor sites found in other animals. These platelets do interact with endotoxin and, through this process, can affect the inflammatory process.

Host factors generated in response to endotoxin can significantly affect platelet function and survival. For example, responses of endothelial cells, leukocytes and macrophages during endotoxemia are responsible for increased levels of factors such as thromboxane, prostacyclin, hydrogen peroxide and other substances which can enhance or reduce platelet aggregation. *Reprint 43. (1/2)*

There is considerable evidence that microvascular injury is significant to the outcome of endotoxemia. However, it is questionable that, particularly in primates, direct endotoxin-platelet interactions are essential to this process.

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